CO_2, H_2O exchange and stomatal regulation of regenerated *Camptotheca acuminata* plantlets during *ex vitro* acclimatization

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Abstract: For finding the changes in CO_2, H_2O exchange and their stomatal regulation during *ex vitro* acclimatization of regenerated *Camptotheca acuminata* plantlets, the net photosynthesis rate (P_n), respiration rate (R_d), light compensation point (L_c) and light saturation point (L_s), transpiration rate (Tr), stomatal conductance (g_s) and water use efficiency(WUE) were measured during 37 days of *ex vitro* acclimatization. The results showed that P_n sharply increased until 29 days, then slightly decreased. A substantial decrease in L_c and a substantial increase of L_s in the former two weeks were observed, indicating the light regime enlargement for effective leaf photosynthesis. Tr and g_s abruptly decreased during the first week then linearly increased until 29days *ex vitro* acclimatization, reflecting the strong regulation effect of stomata on water changes of *ex vitro* acclimating plantlets. Stomatal regulation effect on CO_2 exchange was different from that on water exchange, i.e. P_n was almost independent of g_s during the first week, while P_n was significantly correlated with g_s thereafter (i.e. dual patterns). Different from dual patterns of g_s-P_n relation, the Tr monotonously linearly increased with g_s. Furthermore, WUE was almost independent on g_s during the first week, while a marked decreasing tendency with g_s was found thereafter. At the beginning of the acclimatization, WUE was mainly determined by photosynthetic capacity, while transpiration becomes a main determinant factor for WUE from 7 to 37 days' acclimatization.

Keywords: *Camptotheca acuminate*; Acclimatization; Photosynthesis; Transpiration; Water use efficiency; Relation between stomatal conductance (g_s) and net photosynthesis rate (P_n); g_s-WUE relation

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Introduction

Micropropagation is an important tool for mass production of uniform plants because of its high efficiency, with good phytosanitary status and quality control. Considerable efforts have been directed to optimize the conditions for *in vitro* stages of micropropagation, but the process of acclimatization of micropropagated plants to the soil environment has not fully been studied. Consequently, the transplantation stage continues to be a major bottleneck in the micropropagation of many plants, most species grown *in vitro* requires an acclimatization process in order to ensure that sufficient number of plants survive and grow vigorously when transferred to soil (Hazarika 2003).

Generally, *in vitro* plantlets are photomixotrophical plants. The acquirement for photosynthetic ability is the base for the survival of micropropagated plantlets in open conditions. How plantlets adjust its photosynthetic apparatus during *ex vitro* acclimatization is of scientific significance. As reviewed by Pospíšilová *et al* (1999), photosynthesis adjustment, anatomical structure and water relations can widely be observed during *ex vitro* acclimatization. While to date, information on photosynthetic acclimatization of regenerated *C. acuminata* plantlets during *ex vitro* acclimatization is so limited. We have described a protocol providing the establishment of *in vitro* regeneration of leaf explants and rooting of *C. acuminata* (Wang *et al*. 2005). Comparing to seedlings, moreover, less root-tips of micropropagated plantlets may strongly affect the stomatal function after transfering to open air and soil (Wang *et al.* 2004). For further understanding the acclimatization processes, we designed an experiment to find the variation in CO_2, H_2O exchange and their stomatal regulation of regenerated *C. acuminata* plantlets during *ex vitro* acclimatization in order to optimize culture conditions and obtaining high-quality regenerated plantlets.

Materials and methods

Plant materials

The shoots for rooting in this study were obtained from *in vitro* proliferating shoot cultures of *C. acuminata*. Cultures were maintained by subculturing shoots every five weeks on a shoot multiplication medium, B5 medium supplemented with 0.5 $mg \cdot L^{-1}$ of BA, 20 $g \cdot L^{-1}$ sucrose and 6 $g \cdot L^{-1}$ agar, pH 5.8. The cultures were incubated at 25 ± 2 °C, under a 16-h photoperiod of 40 $\mu mol \cdot m^{-2} \cdot s^{-1}$ irradiance provided by white fluorescent tubes.

C. acuminata plantlets of about 4 cm in height were selected from the glass culture vessels and transferred to the greenhouse. Each plantlet was immediately transferred to small pots containing soilrite and sand (1:1) in the greenhouse. The whole unit was shaded by a clear plastic film for one week. Thereafter, plastic film cover was removed and plantlets began to grow at the greenhouse condition. The light in green house was maximum around 300 $mmol \cdot m^{-2} \cdot s^{-1}$.

Measurements of photosynthetic competence

The photosynthesis measurements were carried out during at 0, 7, 14, 21, 28, 37 days after *ex vitro* transplantation. Measurements were performed on the top with 2–3 leaves (fully ex-

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panded leaves) at each sample date with a Li-6400 portable photosynthesis system (LiCor, Lincoln, NE, USA). Each leaf was a single replication, and there were 10 replications (5 different plants) per sample date (n=10). Light response curves were analyzed by Tamiya equation, which has been described by Wang *et al.* (2001). Three parameters, R_d, L_c and L_s were derived from light response curves. When the P_n is 90% of maximum photosynthesis (when P_n is higher than 1.0μmol·m^{-2}·s^{-1}) or 0.1μmol·m^{-2}·s^{-1} lower than maximum photosynthesis(when P_n is lower than 1.0μmol·m^{-2}·s^{-1}), the light corresponding to this photosynthetic rate is assumed as light saturation point (L_s), while the light compensation point (L_c) is the light density and net photosynthetic rate is zero. Water use efficiency is calculated as the ratio between saturated photosynthetic rate and transpiration rate. Statistical analysis and regression analysis were performed by SPSS11.0 and Excel 2003.

Results and discussion

Changes in photosynthetic-related parameters

Photosynthetic parameters, light-saturated net photosynthetic rate (P_n), stomatal conductance (g_s), transpiration rate (Tr), water use efficiency (WUE) and parameters derived from the light response curves, dark respiration at day time (R_d), light compensation point (L_c), light saturation point (L_s) were listed in Table 1.

Table 1. Changes in light-saturated photosynthetic rate (P_n), dark respiration rate (R_d), light compensation point(L_c) and light saturation point (L_s), stomatal conductance (g_s), water use efficiency(WUE) and transpiration rate (Tr) during the ex vitro acclimatization

Date of *ex vitro* acclimatization	Parameters						
	P_n(μmol·m^{-2}·s^{-1})	R_d(μmol··m^{-2}·s^{-1})	L_c(μmol·m^{-2}·s^{-1})	L_s(μmol·m^{-2}·s^{-1})	g_s(mol·m^{-2}·s^{-1})	WUE(mmol·mol^{-1})	Tr(mmol·m^{-2}·s^{-1})
May 31	0.21(0.2)	1.06(0.09)	116.0(21.0)	276.4(20.5)	0.030(0.018)	0.3(0.1)	1.4(0.6)
Jun 7	1.15(0.34)	0.76(0.24)	23.0(5.6)	580.7(41.5)	0.011(0.002)	2.1(0.6)	0.6(0.3)
Jun 14	2.9(1.0)	0.65(0.24)	18.0(4.8)	1015.2(46.7)	0.050(0.003)	2.7(0.8)	1.1(0.3)
Jun 22	6.8(1.1)	1.23(0.09)	12.1(3.8)	772.1(36.2)	0.109(0.031)	2.1(0.5)	3.4(1.0)
Jun 29	7.8(2.1)	1.20(0.37)	15.1(3.4)	1215.9(49.2)	0.145(0.036)	1.9(0.4)	4.3(0.8)
Jul 7	5.4(1.3)	0.57(0.19)	8.2(2.3)	1021.5(36.5)	0.097(0.019)	3.2(1.0)	1.5(0.5)

With time of *ex vitro* acclimatization, P_n sharply increased until 29days, and then slightly decreased, while no general tendency of R_d was found. Several studies have concentrated on the photosynthetic changes of plantlets during *ex vitro* acclimatization (Van huylenbroeck *et al.* 1998; Pospíšilová *et al.* 1999; Estrada-Luna *et al.* 2001; Rodríguez *et al.* 2003). According to the photosynthetic changes, some phases, such as acclimatization and post-acclimatization phases have been classified (Estrada-Luna *et al.* 2001). In this study, the photosynthetic capacity has reached the maximum state after three to four weeks of acclimatization.

The differences between L_c and L_s show the light regime where the light reaction center do not saturate but can induce net CO_2 absorption. A substantial decrease in L_c and a substantial increase of L_s in the former two weeks were observed, indicating the light regime enlargement for leaf photosynthesis. Similar to our study, a study on sugarcane plantlets has proved that L_s increased with acclimatization time, but the L_c value did not change in their study (Rodríguez *et al.* 2003). The light for their culture condition was constant at ca. 2000μmol·m^{-2}·s^{-1}, while the light in our greenhouse condition was peaked at around 300μmol· m^{-2}·s^{-1}. The low light condition in our study may induce the plantlet trying to exploit the low regime of light for photosynthesis.

Transpiration and stomatal conductance for water are two important parameters for water balance study of plants. During the first week, transpiration rate decreased from 1.4 mmol·m^{-2}·s^{-1} to 0.6 mmol·m^{-2}·s^{-1}, and then linearly increased to 4.3 mmol·m^{-2}·s^{-1} until 29 days, slightly decreased afterward. Similarly, stomatal conductance slightly decreased from 0.030 mol·m^{-2}·s^{-1} at the beginning of the *ex vitro* acclimatization to 0.011 mol·m^{-2}·s^{-1} 7 days later, then steadily increased from 0.011 mol·m^{-2}s^{-1} to 0.145 mol·m^{-2}·s^{-1} at 29the day, and then slightly decreased afterward. This coincidence reflected the strong regulation effect of stomata on water changes of *ex vitro* acclimating plantlets.

Water use efficiency was substantial low at begining of the *ex vitro* acclimatization, while it sharply increased to a steady level of 2.1mmol·mol^{-1} until 7 days of acclimatization. The water use efficiency from June 7 to July 7 kept at a steady level from 1.9 mmol·mol^{-1} to 3.2 mmol·mol^{-1}. These may be related to quickly effect of stomata regulation on water and CO_2 diffusion (Pospíšilová *et al.* 1999), which are important for the survival of plantlets in ambient environment during acclimatization.

Different effect of stomatal regulation on P_n and Tr during *ex vitro* acclimzation

Stomatal control is critical for survival and directly affected the physiological performance of the plantlets. For normal plants, stomatal conductance are generally strongly correlated with both photosynthesis and transpiration rates (Wang *et al.* 2003, Zu *et al.* 2006). *Ex vitro* acclimatization is a process of abnormal plant to normal plant. Until now, less information is available in the difference of stomatal regulation on photosynthesis and respiration. In this study, we found that P_n was almost independent of g_s during the first week (r^2=0.19, p>0.001), while P_n was significantly correlated with g_s one week after acclimatization (r^2=0.90, p<0.001), showing the stomatal regulation differences during *ex vitro* acclimatization. Different from dual patterns of g_s-P_n relation, the transpiration (Tr) linearly increased with g_s during this process (r^2=0.92, p<0.001) (Fig. 1). This finding showed the effects of stomatal regulation on CO_2 exchange were different from that on water exchange.

Sharp decrease in stomatal density and changes in stomatal shape from ring to ellipse during acclimatization to *ex vitro* environment were reported in tobacco and other species (Tichá, 1999). Moreover, regular changes in stomatal conductance during this process were reported and also observed in this study. At the beginning of the *ex vitro* acclimatization, the plantlets was almost heterotrophic plant. Although water can diffuse through the stomata, CO_2 can only diffuse into the leaf in a very limited amount since the unsound photosynthetic machinery. After the buildup of photosynthetic machinery, g_s-P_n relation became sta-

ble.

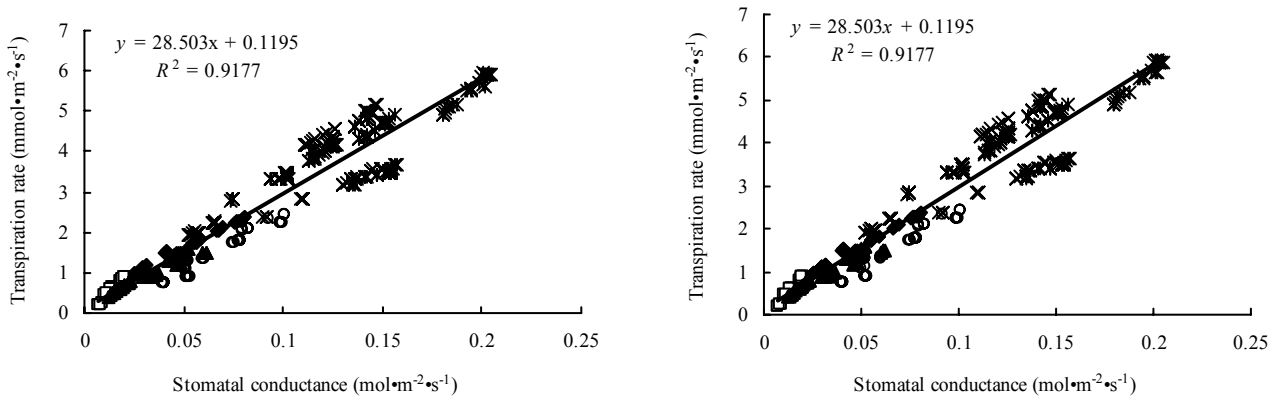

Fig. 1 The influences of stomatal conductance (g_s) on transpiration rate, Tr (Left) and net photosynthetic rate (P_n) (Right) at saturation light (PAR>500μmol·m^{-2}·s^{-1}).

Effect of Stomatal regulation on water use efficiency during *ex vitro* acclimatization

Generally speaking, the stomatal regulation let plants kept at a relative constant efficiency in water utilization. However, different plants even the same plants in different hydrological regime differs in its g_s-WUE relation with the changes in g_s. For example, Zu *et al.* (2006) has reported that *Eupatorium adenophorum*, an alien invasive species, can increase WUE with stomatal conductance decrease when grown in xeric habitats, while WUE increases with stomatal conductance increase in hygric habitats. This water use strategy let this species growth well. As shown in Fig.2, two patterns in g_s-WUE relation have been found in plantlets of *C. acuminata*, i.e. WUE was almost independent of g_s during the first week, while a slight decrease tendency with g_s increase was found thereafter(r^2=0.14, p<0.001). This finding indicated that the effect of stomatal regulation on water utilization was quickly attained by about one week.

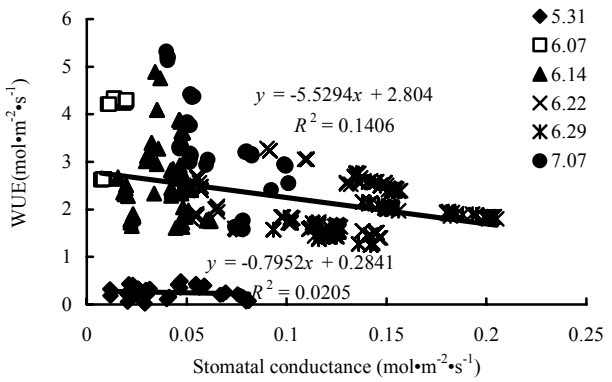

Fig. 2 The influences of stomatal conductance (g_s) on photosynthetic water utilization efficiency (WUE) at saturation light (PAR>500μmol·m^{-2}·s^{-1})

Determinant factors for water use efficiency during *ex vitro* acclimatization, P_n or Tr

As mentioned above, stomata differently regulate photosynthesis and transpiration during *ex vitro* acclimatization (Fig. 1). Moreover, the much lower WUE at the beginning of the acclimatization was independent of g_s, but it significantly correlated with g_s thereafter, showing the dual pattern in g_s-WUE relation (Table 1, Fig. 2). Since WUE is the ratio between photosynthesis and transpiration, one question is which of these two parameters mainly determines the WUE in different phase of acclimatization. As shown in Fig. 3, WUE linearly increased with P_n (r^2=0.44, p<0.001), but almost constant with Tr (r^2=0.02, p>0.1) at the beginning of the acclimatization. One-week acclimatization and thereafter, WUE was almost constant with P_n (r^2=0.02, p>0.1), but exponentially decreased with Tr (r^2=0.29, p<0.001). These findings indicated that at begining of the acclimzation, WUE is mainly determined by photosynthetic capacity, while transpiration becomes a main determinant for WUE from 7 to 37 days of acclimatization.

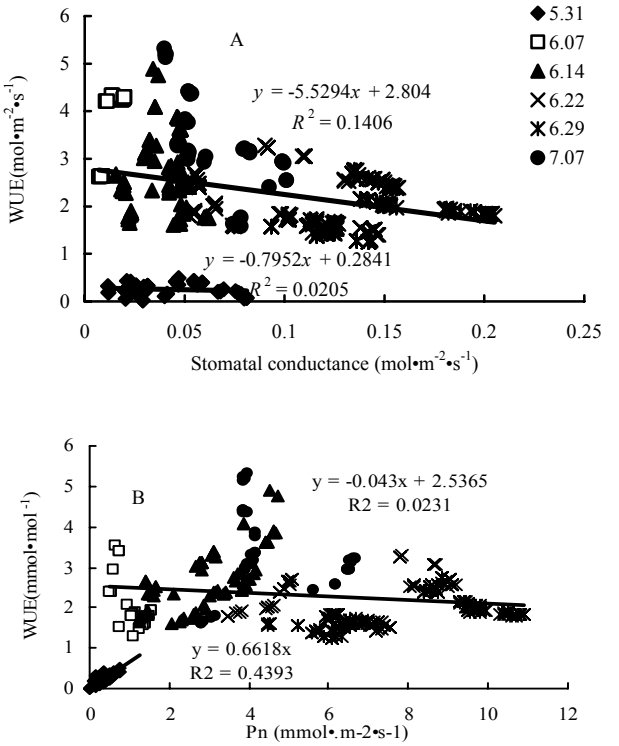

Fig. 3 the influences of photosynthesis (A) and transpiration (B) on water use efficiency of regenerated *Camptotheca acuminata* plantlets during *ex vitro* acclimatization

Conclusions

With time of *ex vitro* acclimatization, P_n sharply increased until 29th day, and then slightly decreased, while no general tendency of R_d was found. A substantial decrease in L_c and a substantial increase of L_s in the former two weeks were observed, indicating the light regime enlargement for leaf photosynthesis. During the first week, transpiration rate and stomatal conductance abruptly decreased then linearly increased until 29days, reflecting the strong effect of stomata regulation on water changes of *ex vitro* acclimating plantlets. Water use efficiency was substantial low at the beginning of the *ex vitro* acclimatization, while it sharply increased to a steady level within 7 days of acclimatization and thereafter.

The effect of stomatal regulation on CO_2 exchange is different from that on water exchange, i.e. P_n was almost independent of g_s during the first week, while P_n was significantly correlated with g_s one week after acclimatization. Different from dual patterns of g_s-P_n relation, the transpiration (Tr) linearly increased with g_s during this process. The effect of stomatal regulation on water utilization is quickly attained by about one week. WUE was almost independent of g_s during the first week, while a marked decreasing tendency with g_s increases was found thereafter. At the beginning of the acclimatization, WUE was mainly determined by photosynthetic capacity, while transpiration becomes a main determinant for WUE from 7 to 37 days of acclimatization.

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